Earlier Flowering Induced by Over-Expression of *CO* Gene Does Not Accompany Increase of Artemisinin Biosynthesis in *Artemisia annua*

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Abstract: **The early flowering gene *CONSTANS (CO)* from *Arabidopsis thaliana* was transferred into *Artemisia annua* using the *Agrobacterium tumefaciens*-mediated transformation system. The plant expression vector pBI*CO* was constructed by inserting the *CO* gene into the binary vector pBI121 under the control of CaMV 35S promoter. Analyses of PCR, PCR Southern blot, and Southern blot revealed that the transgenic plants contained the foreign *CO* gene. The results of RT-PCT and RT-PCR Southern blot suggested that the foreign *CO* gene had expressed at the transcriptional level. Although the flowering time of the *CO* transgenic plant was about 2 weeks earlier than that of the non-transgenic plant under short-day conditions, no significant difference in artemisinin content was found between the flowering transgenic plant and the non-flowering non-transgenic plant. These results show that the usually observed increase of artemisinin content before plant flowering under natural conditions is not a direct consequence of flowering itself, perhaps there is even no direct linkage between flowering and artemisinin biosynthesis.**

Key words: ***CO*** **gene, flowering, *Artemisia annua* L., artemisinin biosynthesis.**

Abbreviations:
6-BA: N^6-benzyladenine
CO: *CONSTANS*
DW: dry weight
fpf1: flowering promoting factor 1
NAA: α-naphthaleneacetic acid

Introduction

Artemisinin, a sesquiterpene lactone endoperoxide, is an effective antimalarial drug obtained from the leaves of the Chinese medicinal herb *Artemisia annua* L. (Cooperative Research Group on Artemisinin Structure, 1977; Liu et al., 1979). This drug is shown to possess considerable inhibitory activity against *Plasmodium falciparum,* the causative parasite of malaria (Klayman, 1979). The aerial parts of *A. annua* contain between 0.01% and 0.5% (dry weight) artemisinin (Wallaart et al., 2000). The relatively low content of artemisinin in the plant has been the limiting factor for the isolation of the substance on a commercial scale (Van Geldre et al., 1997; Dhingra et al., 2000). The total organic synthesis of artemisinin has been achieved, but is very complicated and low yields are obtained (Schmid and Hofheinz, 1983). Moreover, attempts to over-produce artemisinin through cell, tissue, or organ cultures have not been successful (Weathers et al., 1994; Abdin et al., 2003). Presently, the plant is still the only viable source of artemisinin. This reflects the lack of sufficient understanding of artemisinin biosynthesis and its regulation in planta. Therefore, a thorough understanding of the biosynthetic pathway of artemisinin and its regulation may enable us to alter its formation in a direct way, for example by metabolic pathway engineering (Wallaart et al., 2001, Ro et al., 2006).

The main artemisinin content is found in the leaves and flowering tops of the plant. Factors such as plant variety, cultivation condition, locality, and plant developmental stages all influence the artemisinin content of the plant (Paniego et al., 1993; Ferreira et al., 1995). The results of artemisinin content at different developmental stages indicated that the artemisinin biosynthesis increased with a progressive increase in plant growth, reaching a maximum prior to flowering (Lierch et al., 1986; Woerdenbag et al., 1994; Chan et al., 1995). However, it is not known if this is just a coincidence or if some inner linkage between flowering and artemisinin biosynthesis exists. In our previous work, the preliminary results of using *fpf1* as a flower-promoting gene showed that artemisinin biosynthesis does not have any direct linkage with flowering (Wang et al., 2004). In order to further clarify the matter, we studied this linkage at the molecular level by transferring the flower-promoting gene *CONSTANS* of *Arabidopsis thaliana* (Putterill et al., 1995) into *A. annua*. The results showed that the over-expression of the *CO* gene indeed resulted in the early flowering of the transgenic *A. annua*. However, no significant differences in artemisinin content were found between the flowering transgenic plant and the non-flowering non-transgenic plant. Our work was of both theoretical and practical significance. In theory, the results can provide experimental evidence for the regulation of artemisinin biosynthesis, and in practice, it can provide theoretical guidance for choosing a suitable harvesting stage for higher production of artemisinin.

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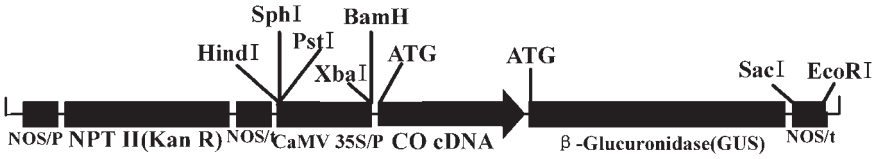

Fig. 1 Diagram of the recombinant plasmid pBI*CO*.

Materials and Methods

Plant material and culture conditions

The high artemisinin-yielding strain 001 of *A. annua* was collected from Sichuan Province in China. The seeds were surface-sterilized with 0.1% mercuric chloride for 10 min, followed by washing with sterile distilled water. Then the seeds were germinated on MS medium supplemented with 3.0% sucrose and solidified with 0.7% agar. The pH was adjusted to 5.8 – 5.9 before the addition of agar. The medium was autoclaved at 1 bar for 20 min. The cultures were maintained at 26 °C under 16-h photoperiods.

Construction of plasmids

The plasmid pBSK*CO* containing the *CO* gene (Putterill et al., 1995) was digested with *Hin*dIII and blunted with T4 DNA polymerase, then digested with *Bam*HI to obtain a 1.1-kb fragment containing the complete open reading frame. This 1.1 kb fragment was then inserted into the *Xba*I (blunted with Klenow large fragment) and *Bam*HI sites of plasmid pBI221 to form the intermediate vector pBI221-*CO*. The intermediate vector pBI221-*CO* was digested with *Sac*I and *Bam*HI, and the 2.9-kb fragment containing the chimeric *CO* gene was collected, then inserted into the *Sac*I-*Bam*HI sites of the binary vector pBI121 to generate recombinant binary vector pBI*CO* (Fig. **1**). The recombinant binary vector pBI*CO* contained both the NPT II gene of kanamycin resistance driven by the NOS promoter and the *CO-GUS* fusion gene driven by cauliflower mosaic virus (CaMV) 35S promoter. *A. annua* is very sensitive to kanamycin, so kanamycin was used for the selection of the transformants.

The plant expression vector containing the recombinant plasmid pBI*CO* was introduced into the disarmed *Agrobacterium tumefaciens* strain LBA4404 by triparental mating.

Transformation and regeneration of transformants

The transformation of *A. annua* was accomplished according to Chen et al. (1999). Young leaves of the seedlings subcultured for 3 weeks were used for the transformation. Prior to infection, the inoculum of *A. tumefaciens* was grown in 50 mL of liquid YEB medium (containing 100 mg/L rifampicin, 50 mg/L streptomycin, and 50 mg/L kanamycin) at 28 °C under agitation to an OD_{600} of 0.6 – 0.8. Then the bacterial suspension was pelleted by centrifugation, resuspended in 20 mL of fresh YEB liquid medium (without antibiotics) containing 100 µM acetosyringone and cultured for another 2 h under the same conditions for further activation. The activated bacterial suspension was diluted 10-fold with MS liquid medium and was used for *A. annua* transformation. The wounded leaves were immersed in the diluted bacterial suspension for 20 min, blotted on sterile filter paper, and transferred to solid MS medium, incubated in the dark at 28 °C for 3 days. After 3 days co-cultivation, the leaves were transferred to shoot-inducing medium (MS basal medium supplemented with 2.5 mg/L 6-BA, 0.05 mg/L NAA, 500 mg/L carbenicillin, and 15 mg/L kanamycin) to induce shoots. Carbenicillin was used to eliminate surplus *A. tumefaciens*, kanamycin was used to select the transformants, and the media without kanamycin were considered as controls. The explants were transferred to fresh shoot-inducing medium at weekly intervals during the first month. Afterward, subcultures were made every 2 weeks. The concentration of carbenicillin was reduced to 100 mg/L after 8 weeks, at 12 weeks carbenicillin was completely removed from the medium. When the induced shoots had grown to about 2 – 3 cm in height, the shoots were separated from the explants and transferred to root-inducing medium (MS basal medium supplemented with 0.05 mg/L NAA and 15 mg/L kanamycin) to induce roots.

PCR analysis for transformed plants

DNA isolated from putatively transformed plants and non-transformed plants was used as template. PCR amplifications were conducted using the *CO* gene primers and the *CO-GUS* fusion gene primers. The primers used to amplify the *CO* gene were: forward 5′-GGT CAA ACG CCT GCA CCG TGT A-3′, reverse 5′-TCT TTG CGA ACC GGC CAT TGA C-3′. The PCR condition was: 94 °C for 40 s, 68 °C for 1 min, 72 °C for 1.5 min, 30 cycles, finally 72 °C for 10 min. The reverse primer designed for the *CO-GUS* fusion gene was: 5′-CAA AGC CAG TAA AGT AGA ACG GT-3′, the forward primer was the same as that in the above amplification of the *CO* gene. The *CO-GUS* fragment between the two primers was 2.0 kb. The amplification conditions for the *CO-GUS* fusion gene consisted of 30 cycles: 40 s at 94 °C, 1 min at 65 °C, 1.5 min at 72 °C per cycle, and finally followed by a further 10-min extension step at 72 °C.

The amplified *CO* and *CO-GUS* fragments were electrophoresed on 0.8% agarose gel and photographed using the BioCaptMW software in a VILBER LOURMAT gel image system. After that, the fragments were transferred to positively charged nylon membrane (Roche), and PCR Southern analyses were carried out using [α-^{32}P]-radioactively labelled *CO* and *CO-GUS* fragment as a probe.

Southern blot analysis

Southern blot analysis was carried out according to Sambrook et al. (1989). The total genomic DNA from putatively transformed plants and non-transformed plants was digested with *Eco*RI and *Bam*HI, DNA from plasmid pBI*CO* was also digested with these same two enzymes as a positive control. The completely digested DNA was separated by 0.7% agarose gel electrophoresis and transferred onto nylon membrane (Roche). Southern hybridization was performed using [α-^{32}P]-radioactively labelled *CO-GUS* fragment as a probe. The membrane

was washed at high-stringency washing conditions in a buffer consisting of 0.1 × SSC and 0.5% SDS at 65 °C.

RT-PCR analysis

Total RNA from the leaves of transgenic plants and control was isolated by a one-step method using Trizol extract buffer (Invitrogen). First-strand cDNA synthesis was performed on total RNA treated with DNase I (Roche) using a TaKaRa RNA PCR Kit (TaKaRa). The protocol for reverse transcription was: 42 °C for 30 min, 99 °C for 5 min, and 5 °C for 5 min. The resultant first-strand cDNA was used as a template to amplify the *CO* gene. The primers used here were the same as those in the PCR analysis in Section "PCR analysis for transformed plants".

The conserved tubulin gene was used as an internal control for gel loading. The primers used for amplifying the tubulin gene were: forward 5′-AAC AAC TGG GCC AAG GG-3′, reverse 5′-TCC TGG TAC TGC TGG TAC TC-3′. The tubulin gene fragment between the two primers was 1.0 kb. The PCR consisted of 30 cycles: 1 min at 94 °C, 40 s at 65 °C, 1 min at 72 °C per cycle, and the last cycle was terminated with a final 10-min extension step at 72 °C.

The RT-PCR products were separated on 0.8% agarose gel and photographed. The RT-PCR Southern analysis was carried out using [α-^{32}P]-radioactively labelled *CO* gene as a probe. [α-^{32}P]-radioactively labelled tubulin gene was used as an internal control for gel loading and transferring.

The induction of flowering

The rooted germ-free transgenic plants and control were transplanted to a greenhouse for a 3-month growth period under a 16-h photoperiod at 20 °C, then some transgenic plants and non-transgenic plants were treated with an 8-h photoperiod to induce flowering, and others still growing under the 16-h photoperiod were used as the control for flowering.

Detection of artemisinin

The detection of artemisinin was performed according to the method of Zhao and Zeng (1986). The leaves of the non-flowering plants and the leaves as well as the flowers of the flowering plants were collected and dried to constant weight in an oven at 50 °C. Then, the dried leaves and flowers were ground to powder. Some 100 mg of the exactly weighed powder were added to an extraction bottle containing 20 mL of petroleum ether (boiling range 30 – 60 °C) and extracted overnight. After treatment in an ultrasonic bath for 5 min, the extraction mixture was filtered under vacuum. The filtrates were collected and evaporated to dryness. The residue was dissolved in 1 mL methanol and centrifuged at 12 000 rpm for 10 min to precipitate the undissolved components. The supernatant containing the artemisinin extract was used for HPLC detection.

200 µL of the above-prepared artemisinin extract were placed in a 10-mL tube with 800 µL methanol, 4 mL 0.2% sodium hydroxide, mixed and then incubated at 50 °C for 30 min. After cooling to room temperature, 0.5 mL of the reaction mixture was placed in a 1.5-mL Eppendorf tube to which 100 µL methanol and 400 µL 0.05 M acetic acid were added and mixed before purification by filtering on an NC filter (40 µm). The artemisinin standard solutions with concentrations of 4, 8, 12, 16, 20, 24 (µg/mL) were prepared in the same way as the sample.

HPLC was performed using a C_{18} reverse-phase column (4.6 × 250 mm, 5 Micron). A 10-min isocratic programme was used. The mobile phase was 55% 0.01 M sodium phosphate buffer (pH 7.0) and 45% methanol, flow rate was 1 mL/min, the injection volume was 10 µL. The retention time of artemisinin reference was about 4 min under these conditions.

Results and Discussion

Transformation of A. annua *and regeneration of the transformed plants*

The *Agrobacterium tumefaciens* strain LBA4404 harbouring the binary vector pBI*CO* was used for *A. annua* transformation. The plant expression vector pBI*CO* contains the plant-selective marker neomycin phosphotransferase II (NPT-II) gene which makes the transformed cells resistant to kanamycin. *A. annua* transformation was performed essentially according to the method described by Chen et al. (1999). In order to get high shoot-inducing frequency, we optimized the composition of the shoot-inducing medium. With a combination of 0.05 mg/L NAA and 2.5 mg/L BA, the highest shoot-inducing frequency was reached. The *A. annua* shoot induction was very sensitive to kanamycin. When the leaves were cultured on shoot-inducing medium supplemented with 15 mg/L kanamycin, the wounds of the untransformed leaves did not generate shoot clusters, the leaves withered and became yellow, and finally died. However, for the transformed leaves, first, the wounds of the leaves generated some green shoot primordia, later, the shoot primordia spread and generated shoot clusters. Most of the shoots were directly generated from the wounds on the leaves, there were also some leaves which first formed calluses in the wounds, then some were induced to form shoots after modifying the hormone component of the medium, but the shoot-inducing capacity was low. Therefore, it seems best to induce shoots directly from explants rather than from induced calluses. This is in accordance with the observations of Vergauwe et al. (1996) and Han et al.(2005). The well-grown kanamycin-resistant shoots were separated from the explants and transferred to root-inducing medium to induce roots. About 2 weeks later, the shoots rooted in the root-inducing medium. Shoots rooted in medium containing 15 mg/L kanamycin were considered to be transgenic plants.

PCR analysis

PCR amplification was conducted on the lines that were putatively transformed with *CO* gene by their resistance to kanamycin. The amplified product using *CO* gene primers showed a 1.1-kb band, and the size of the band was the same as that of pBI*CO* used as a positive control, but the non-transformed control has no target band. PCR Southern analysis confirmed that the 1.1-kb band was indeed the band representing the *CO* gene (data not shown). The amplified product using *CO* forward and *GUS* reverse primers showed a 2.1-kb band, the size of the band was the same as that when pBI*CO* was used as a positive control, and PCR Southern analysis confirmed that the 2.1-kb band was the band representing the *CO-GUS* fusion gene (data not shown).

Southern blot analysis

Part of the PCR positive transformed plants were further selected for Southern blot analysis. Genomic DNA digested with *Eco*RI and *Bam*HI and hybridized with the *CO-GUS* probe produced bands representing the DNA fragment containing the *CO-GUS* fusion gene. No hybridization bands were present in the untransformed plants (Fig. **2**). The result of the Southern blot showed that the *CO* gene had been integrated into the genome of the transgenic *A. annua* plant.

RT-PCR analysis

Southern blot positive transgenic plants were selected for RT-PCR detection. The amplified product using *CO* gene primers showed a 1.1-kb band in the transgenic plants, but no such band was detected in the non-transformed control. The results of RT-PCR Southern analysis using *CO* gene as a probe confirmed that the 1.1-kb band truly represented the *CO* gene (data not shown). When using tubulin gene primers for the amplification of tubulin gene as an endogenous control, both the transformed plants and the control showed a 1.0-kb band, the RT-PCR Southern analysis using tubulin gene as a probe showed that the 1.0-kb band was indeed the conserved tubulin gene (data not shown).

The CO *transgenic plants flowered earlier than the control*

A. annua is a short-day plant whose inductive photoperiod is 13.31 h (Ferreira et al., 1995). In order to induce *A. annua* flowering, some of the *CO* transformed and non-transformed plants were treated with 8 h/16 h (light/dark) photoperiod, and those still growing at 16 h/8 h (light/dark) photoperiod were used as controls for flowering. It was observed that after short-day treatment for 20 days, the *CO* transgenic plants generated flowering buds, but the non-transformed plants were still in vegetative growth at this time. About 15 days later, the non-transformed plants began to generate flowering buds. In other words, the flowering time of the *CO* transgenic plants was about 15 days earlier than the non-transgenic control. The results showed that the foreign *CO* gene can promote flowering in *A. annua*. This is consistent with the results from *A. thaliana* (Putterill et al., 1995). Both the transformed and non-transformed plants maintained under long days were in vegetative growth during the whole experimental period. This is also consistent with the fact that *A. annua* is a short-day plant (Ferreira et al., 1995).

No direct linkage between flowering and artemisinin biosynthesis

The over-expression of the *CO* gene in *A. annua* successfully induced the early flowering of the *CO* transgenic plants. The results showed that the flowering time of *A. annua* can indeed be regulated by the foreign flower-promoting gene. The objective of this work is to study the possible linkage between flowering and artemisinin biosynthesis. In order to detect whether the artemisinin content varied with the plants' flowering time, the leaves as well as the flowers of the short-day-treated plants (*CO* transgenic plants and non- transgenic plants), and only the leaves of the non-short-day-treated plants (*CO* transgenic plants and non-transgenic plants) were harvested at different developmental stages (vegetative growth period, before flowering, full flowering, and post-flowering) to detect the artemisinin content.

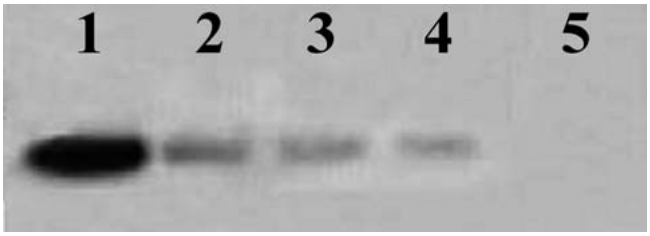

Fig. 2 Southern blot analysis of *CO* transgenic plants. (1) pBI*CO* digested with *Eco*RI and *Bam*HI (+ CK); (2 – 4) DNA from *CO* transgenic plants (lines *CO*-1, *CO*-2, and *CO*-4); (5) DNA from non-transformed plant (– CK). The plant DNA was also digested with *Eco*RI and *Bam*HI.

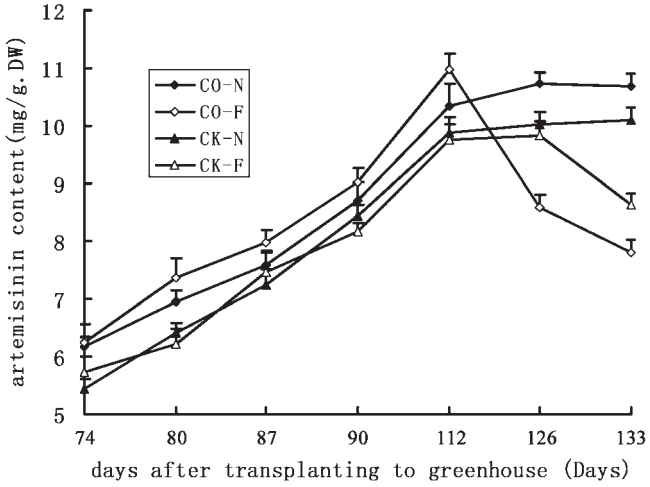

Fig. 3 Comparison of artemisinin content in flowering and non-flowering *CO*-transgenic and CK plants. *CO*-F: flowering *CO*-transgenic plants; *CO*-N: non-flowering *CO*-transgenic plants; CK-F: flowering CK (non-transformed) plants; CK-N: non-flowering CK (non-transformed) plants.

The artemisinin content of different developmental stages is shown in Fig. **3** (each value represents the mean of five samples). From Fig. **3**, we can see that before flowering, the artemisinin content of either the short-day-treated *CO* transgenic and non-transgenic plants, or the non-short-day-treated plants, followed a definite trend and increased with a progressive increase in plant growth. The artemisinin content of the short-day-treated *CO* transgenic plants reached its maximum before the emergence of flowering buds, the short-day-treated non-transformed plants, although in vegetative growth, had an artemisinin content close to this maximum value at the same time. The plants (both transformed and non-transformed) treated with a 16 h/8 h (light/dark) photoperiod, remained in vegetative growth, but their respective artemisinin content showed no significant differences in comparison with those of the short-day-treated plants. After flowering, the artemisinin content of the plants which had flowered (*CO* transgenic plants and non-transgenic plants) began to decrease significantly, but for the non-short-day-treated *CO* transgenic plants and control plants, which were still in vegetative growth, their artemisinin content remained at a high level.

The above results showed that the foreign *CO* gene can indeed promote *A. annua* to flower earlier, but since the artemisinin content of the flowering transformed plants and that of the non-transformed control plants did not manifest significant differences, we can conclude that the increase of artemisinin

content before flowering is not necessarily induced by flowering itself, perhaps it is just a time coincidence, and this was also observed in the results with flower-promoting factor 1 *(fpf1)* (Wang et al., 2004). The reasons underlying this could be that, in the later vegetative period, the plant has stored plentiful material for artemisinin biosynthesis and the glandular trichomes, where artemisinin is synthesized and stored, are well developed and have the highest artemisinin biosynthesis capacity, so the artemisinin synthesis reaches its maximum. In conclusion, based on the above results, we consider that there is no evidence for a direct linkage between flowering and artemisinin biosynthesis.

Finally, the possible causes of the observed significant decline in artemisinin synthesis (Fig. **3**) after flowering are: artemisinin is produced in the glandular trichomes on the leaf surface, as leaves senesce and wither after flowering, the biosynthesis capacity of the trichome in the senescent leaf declines; trichomes of senescent leaves normally burst and release their contents (Duke and Paul, 1993), and this also contributes to the decline in artemisinin content. This study showed that the artemisinin content reached a maximum before flowering, when the plant has almost reached its full size and possessed the highest leaf mass and area. Based on the results, *A. annua* plants should be harvested between the later vegetative growth stage and before appearance of flower buds to obtain maximum artemisinin production. In fact, experienced farmers in China already choose this period for harvesting leaves of *A. annua*.

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